

# Monitoring Human Exposure to Urban Air Pollutants

by R. Barale,<sup>1</sup> I. Barrai,<sup>1</sup> I. Sbrana,<sup>2</sup> L. Migliore,<sup>2</sup>  
A. Marrazzini,<sup>1</sup> V. Scarcelli,<sup>2</sup> E. Bacci,<sup>2</sup> A. Di Sibio,<sup>2</sup>  
A. Tessa,<sup>2</sup> L. Cocchi,<sup>2</sup> V. Lubrano,<sup>2</sup> C. Vassalle,<sup>2</sup> and  
J. He<sup>3</sup>

A multidisciplinary study on a general population exposed to vehicle exhaust was undertaken in Pisa in 1991. Environmental factors such as air pollution and those associated with lifestyle were studied. Meanwhile, biological and medical indicators of health condition were investigated. Chromosomal aberrations, sister chromatid exchanges (SCEs), and micronuclei in lymphocytes were included for the assessment of the genotoxic risk. Because of the large number (3800) of subjects being investigated, standardization of protocols was compulsory. The results on data reproducibility are reported. To assess the reliability of the protocol on a large scale, the population of Porto Tolle, a village located in northeast Italy, was studied and compared to a subset of the Pisa population. Preliminary results showed that probable differences between the two populations and individuals were present in terms of SCE frequencies. The study was potentially able to detect the effects of several factors such as age, smoking, genetics, and environment. The *in vitro* treatment of lymphocytes with diepoxybutane confirmed the presence of more responsive individuals and permitted us to investigate the genetic predisposition to genetic damage. The possible influence of environmental factors was studied by correlation analyses with external exposure to air pollutants as well as with several lifestyle factors.

## Introduction

A joint project of the Italian National Research Council (CNR) and the Italian Electric Power Authority (ENEL) aimed at assessing the human risk from exposure to airborne pollutants was undertaken in January 1991 (1). Two samples from the general population were adopted for longitudinal studies; one in the rural area of the Po River delta, Porto Tolle (northeast Italy), where a large thermoelectric plant operates, the other in the urban and suburban area of Pisa (central Italy), mostly polluted by traffic exhaust.

The aim of this report is to introduce the project and give the preliminary results of the cytogenetic study. The project is the result of a complex multidisciplinary design involving epidemiology, lung diseases, immunology,

allergy, biochemistry, genetics, cytogenetics, environmental chemistry, and computer science. External exposure was measured by monitoring CO<sub>2</sub>, SO<sub>2</sub>, NO<sub>x</sub>, O<sub>3</sub>, polyaromatic hydrocarbons (PAHs), and airborne particulate matter. Internal exposure was assessed by evaluating benzo[a]pyrene (BaP) and benzene adducts to hemoglobin and effective dose by BaP adducts to lymphocyte DNA.

The cytogenetic study provides information on the lesions induced by environmental pollutants or by other factors and may possibly allow evaluation of genetic and cancer risks (2). In particular, chromosome aberrations (CA), sister chromatid exchanges (SCEs), and micronuclei (MN), which are considered as important genetic end points and indicators for exposure to mutagens/carcinogens, were analyzed in peripheral lymphocytes in 3800 subjects enrolled in the study. Because numerous households were involved, it is possible to evaluate the influence of genetic makeup on damage expression. Genetic heterogeneity of the population versus homogeneity within families was quantified by studying 10 polymorphic loci in erythrocytes. Moreover, lymphocytes were treated *in vitro* with diepoxybutane (DEB) to determine individual sensitivity to SCE induction (3). A detailed questionnaire, including 120 items about lifestyle and occupation also allows assortment of environmental factors. Lymphocytes

<sup>1</sup>Dipartimento di Biologia Evolutiva, Università di Ferrara, 44100-Ferrara, Italy.

<sup>2</sup>Dipartimento di Scienze dell'Ambiente e del Territorio, Università di Pisa, 56100-Pisa, Italy.

<sup>3</sup>Department of Environmental Health, Zhejiang Medical University, Hangzhou, People's Republic of China.

Address reprint requests to R. Barale, Dipartimento di Biologia Evolutiva, Università di Ferrara, 44100-Ferrara, Italy.

were also treated with  $\alpha$ -naphthoflavone, which has been shown to increase the basal SCE levels of individuals exposed to nonmutagenic carcinogens such as tetrachloro-dibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs) (4). Blood samples collection, processing, and slides preparation are expected to be complete at the end of 1993. Here we provide the preliminary results on SCE and MN frequency in 120 subjects belonging to both populations on the bases of standardized procedures.

## Material and Methods

Human subjects between 8 and 73 years of age were selected on a household basis. Socioeconomic status, age, and sex were taken into account to obtain representative samples of the general population. The Porto Tolle sample consisted of 60 persons and was mainly used to standardize and adopt a detailed protocol for all procedures such as blood sampling, lymphocyte culturing and processing, and slide preparing, staining, and scoring. The Pisa sample (3800 people) was selected according to the same criteria; here we present the results of cytogenetic analyses on 60 subjects. In Table 1, a description of the two samples is given. The distribution by sex in both samples was not significantly different ( $\chi^2 = 0.13$ ). The distribution of smokers according to location was not significant in either males or females ( $\chi^2 = 0.126$  and  $1.031$ , respectively, both with a 1 df). The proportion of female smokers was less than male smokers, although not significant ( $\chi^2 = 2.944$ ). The oldest group was constituted by female smokers in Pisa (55.0 years); the youngest group was male smokers in Porto Tolle (34.9 years). It should be noted that the Porto Tolle sample was younger than the Pisa sample ( $t = 2.138$ ,  $p = 0.034$ ).

Due to the length (3 years) of the study and the number of cytogeneticists involved (20 persons), several standardized procedures were established. We found that a large amount of culture media could be distributed into single culture tubes, frozen, and stored at  $-80^\circ\text{C}$  until use, without any difficulty. Indeed, a significantly ( $p < 0.008$ ) higher mitotic index was observed using frozen media, regardless of the media storage time. The possible effect of blood sample storage time (up to 74 hr) at room or  $+4^\circ\text{C}$  temperature was studied in 20 subjects, taking into consid-

eration lymphocyte proliferation, MN, and SCE frequency. No differences were found between 24 and 48 hr, but a higher SCE frequency and a decreased proliferation index was observed at 72 hr. Therefore, blood cultures were initiated as soon as possible with some exceptions (within 24–48 hr) and recorded for possible future controls. Heparinized whole-blood sample (0.3 mL) was added to 4.7 mL of Ham's F10 medium (ICN, Irvine, CA) supplemented with 10% fetal calf serum (ICN, Irvine, CA), with 1.5% phytohemagglutinin (PHA; Wellcome, Pomezia, Italy) and antibiotics (100 IU penicillin and 100  $\mu\text{g}$  streptomycin/mL, Sigma, St. Louis, MO). Two independent cultures were set up for CAs, SCEs, MN scoring and DEB (6  $\mu\text{g}/\text{mL}$ ), and  $\alpha$ -naphthoflavone (40  $\mu\text{M}$ ) *in vitro* treatment for a total of 10 independent cultures per subject. Slides for CAs, SCEs, and MN scoring were prepared following standard procedures.

5-Bromo-2'-deoxyuridine (Sigma) used for the analysis of SCEs was added to cultures (9  $\mu\text{g}/\text{mL}$ ) for the whole incubation period of 72 hr. For CAs analysis, the cultures were incubated at  $37^\circ\text{C}$  for 48 hr and 72 hr for MN analysis.

Colchicine, 4  $\mu\text{g}/\text{mL}$  (Sigma), was added 2.5 hr before harvesting time for CAs and SCEs analysis. Metaphases were prepared according to conventional methods, and slides were stained by using Giemsa staining and a modified fluorescence-plus-Giemsa methodology, respectively.

MN were analyzed in cells whose cytokinesis had been blocked by cytochalasin B (3  $\mu\text{g}/\text{mL}$ ; Sigma), 28 hr before harvesting. Cells were then processed according to the method described by Fenech and Morley (5) and modified to enable use of whole-blood cultures. DEB (6  $\mu\text{g}/\text{mL}$ ) was added to two independent cultures; 50 metaphases and 2000 binucleated cells were scored for the presence of SCE and MN, respectively.

## Statistics

The analysis of SCE frequency per cell was conducted on actual numbers or on appropriate transforms to normalize distributions ( $\sqrt{\text{SCE}}$ ). The package STATGRAFICS (Plus Ware, Rockville, MD) was used for statistical analysis and graphics production.

The SCE count per cell, independent of subject, was used to define the threshold value for high frequency cells (HFC) (6). It was then possible to classify the individuals according to the number of cells above threshold.

## Results

### Effectiveness of Standardization

Independent cultures, slide preparation, and scoring were considered the main possible sources of errors. Therefore, their quantification and possible reduction was compulsory in this broad study. Eight trained scorers analyzed 25 metaphases in two different slides from the same donor, and the results are represented by a multiple box and whisker plot in Figure 1A. Two-way ANOVA showed no differences either among scorers or slides ( $F = 0.539$ ,  $p = 0.80$ ;  $F = 2.179$ ,  $p = 0.14$ , respectively). Figure 1B

Table 1. Numbers of individuals forming the two samples according to sex, average age (years  $\pm$  SD), and smoking habits.

Sex	Porto Tolle		Pisa	
	Number	Age	Number	Age
Males				
Nonsmokers	17	39.4 $\pm$ 18.7	17	39.6 $\pm$ 17.3
Smokers	11	34.9 $\pm$ 14.1	9	45.9 $\pm$ 17.0
Total	28	37.6 $\pm$ 16.7	26	41.8 $\pm$ 17.5
Females				
Nonsmokers	23	40.2 $\pm$ 17.5	28	46.4 $\pm$ 17.0
Smokers	9	39.1 $\pm$ 13.3	6	55.0 $\pm$ 9.0
Total	32	39.9 $\pm$ 16.2	34	47.9 $\pm$ 16.3
Total	60	38.8 $\pm$ 16.5	60	45.4 $\pm$ 17.1

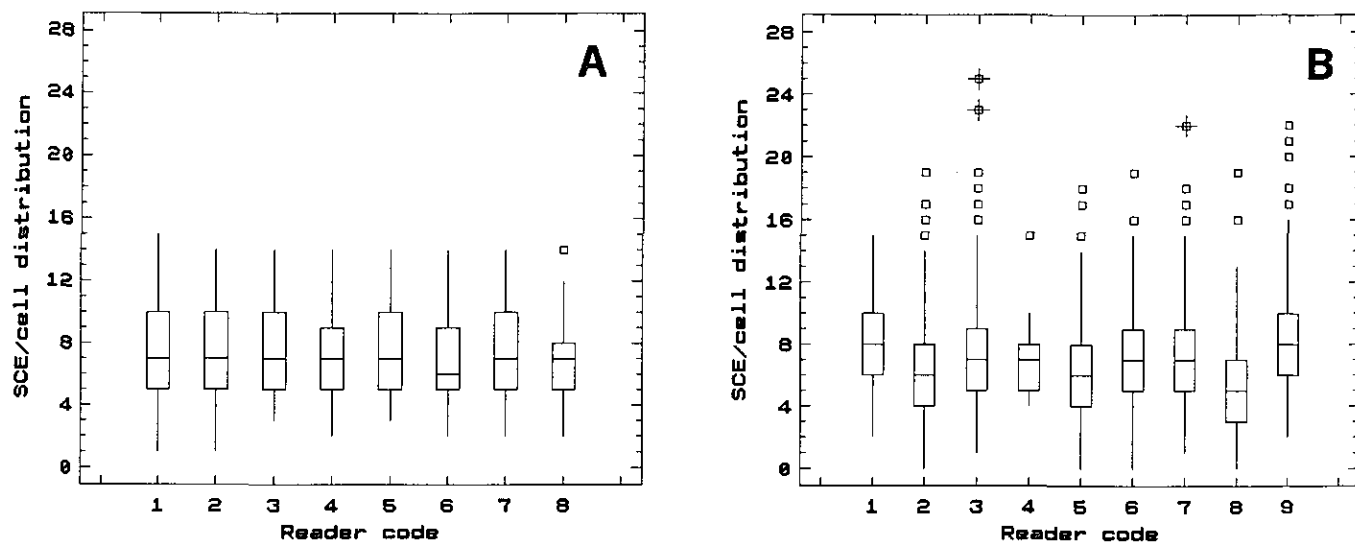


FIGURE 1. (A) Multiple box and whisker plot of sister chromatid exchanges (SCE)/cell distribution according to readers of slides. Each reader scored 50 cells on two slides from the same donor. (B) Distribution of SCE/cell of the Porto Tolle population scored by nine readers; each of them scored slides from six to seven subjects. Points between 1.5 and 3.0 interquartile ranges from the first or third quartile appear as small open boxes, whereas points beyond 3.0 interquartile ranges appear as a cross.

shows the distribution of SCE/cell from 60 subjects scored by 9 readers. The difference observed was due to subject variation.

A similar picture was observed for micronuclei. ANOVA indicated that the variability between readers was not significant ( $F = 1.861$ ,  $p = 0.14$ ), nor was variability among slides ( $F = 0.017$ ,  $p = 0.98$ ). After several trials, we adopted the following scoring procedure: SCE and CA: two slides, each from two independent cultures, were scored by two different readers in a total of 50 and 200 cells, respectively; MN: four different slides (two from each culture) were scored for the presence of MN in 500 binucleated cells by four readers each for a total of 2000 cells.

### Porto Tolle Study

The average SCE frequency/cell was 6.92 (SE = 0.059), lower than the variance (10.37). Its distribution is shown in Figure 2A, and the right skewness (17.42) indicates the presence of HFC cells. In Figure 2B, the distribution of average SCE/individual was reported (6.92, SE = 0.17;  $\chi^2$  test for normality = 5.17, 4 df,  $p = 0.27$ ). In 50 cells from one subject, we expect 5 (HFC) cells to have 13 (95th percentile) or more SCE/cell (6). We found 4 outlier individuals with more than 5 HFCs. The most extreme outlier had 13 cells out of 50 with more than 13 SCE/cell.

Sex had a significant effect on SCE frequency per cell, and women showed a higher number of SCE (7.09, SE = 0.082) than men (6.78, SE = 0.084). The difference was significant ( $t = 2.128$ ,  $p = 0.033$ ). Age had a stronger effect because women were older than men. The sex effect could be reasonably attributed to age. In the subset of

males, the effect of smoking was of borderline significance ( $t = 1.944$ ,  $p = 0.052$ ), but in the subset of the females the effect was considerable ( $t = 5.910$ ,  $p < 0.0001$ ).

The distribution of individuals according to MN frequency is displayed in Figure 3A. The average was 3.97, SE = 0.35, and right skewness was 3.5. We observed that the transformation of  $\sqrt{\text{MN}}$  gave a normalization of the distribution ( $\chi^2 = 7.15$  df,  $p = 0.21$ ). In Figure 3B, the plot of MN on age is given. The slight MN increase observed was not significant.

### Pisa Study

So far, blood samples from more than 500 subjects have been processed for slide preparation, but due to the length of reading, we only had data suitable for statistical analysis for 60 subjects for SCE and MN and for 27 subjects for the DEB *in vitro* treatment. In Figure 2C, the distribution of total SCE/cell is reported. The average is 5.89 (SE = 0.05), variance 8.19, and right skewness 22.6, indicating a higher dispersion of cell with high SCE counts/cell in Pisa population than that in the Porto Tolle population even if its average was lower than Porto Tolle population's ( $t = 4.527$ ,  $p < 0.0001$ ). In Figure 2D, the distribution of mean SCE per individual is reported. The standard deviation was 1.13, lower than that in Porto Tolle (3.22), but the test for normality indicated significant deviation ( $\chi^2 = 15.5$ , 5 df,  $p = 0.008$ ).

Sex had a significant effect on SCE/cell, women showing a lower number of SCE (mean = 5.75, SE = 0.06) than men (mean = 6.06, SE = 0.08). The difference was significant ( $t = 2.92$ ,  $p = 0.0035$ ). Age had a strong effect ( $F = 8.54$ ,  $p < .0001$ ). Because women were older than men, the

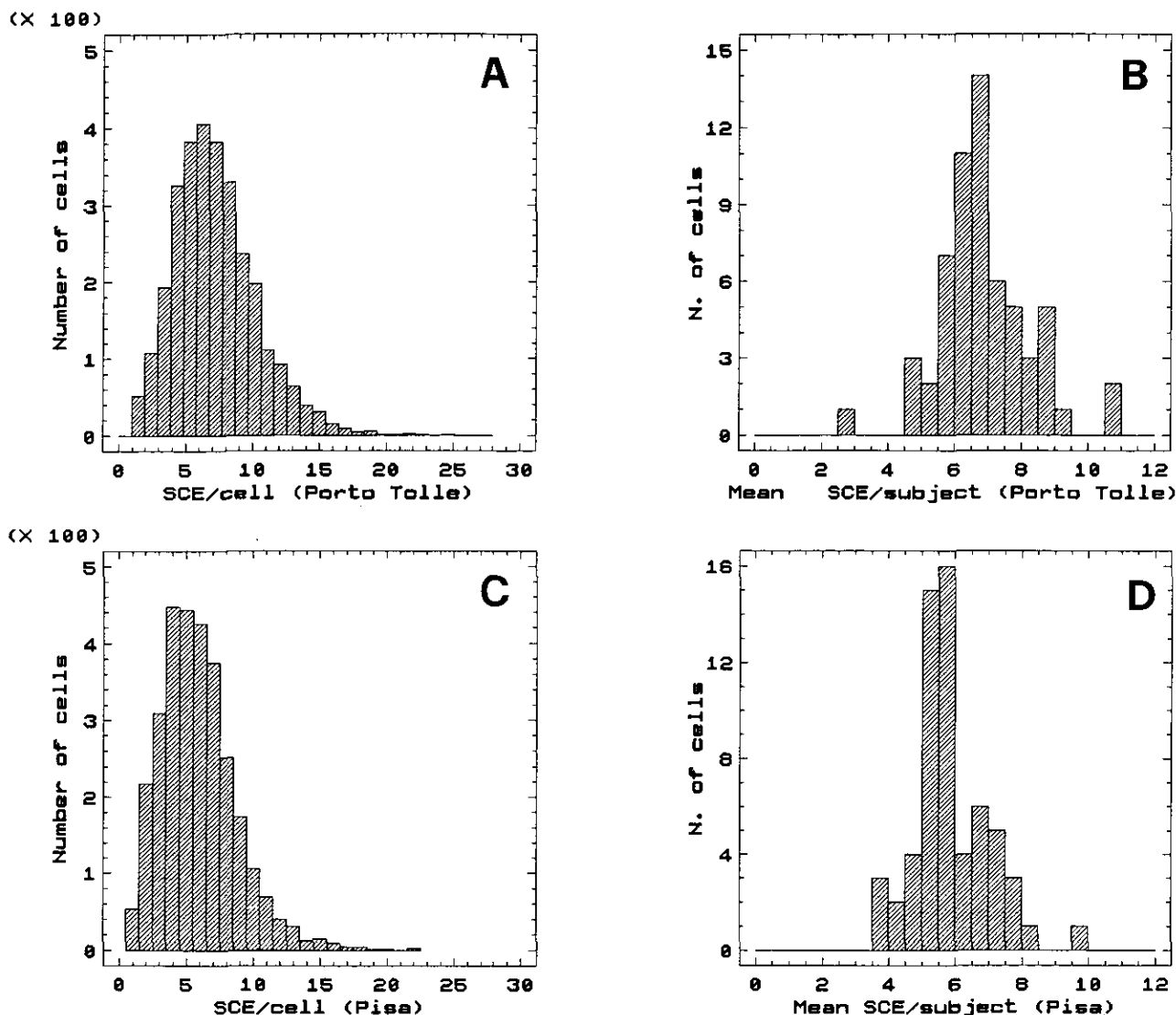


FIGURE 2. Distribution of sister chromatid exchange (SCE)/cell in Porto Tolle (A) and Pisa (C) populations. Distribution of mean SCE of individuals in Porto Tolle (B) and Pisa (D).

sex effect could not be reasonably attributed to age. The effect of smoking was not significant in either males or females. ANOVA multifactor analysis will be performed on a larger set of data.

The distribution of individuals according to MN frequency is displayed in Figure 3C. The average was 4.20 (SE = 0.037) and right skewness was 3.3. We observed that the transformation of MN did not normalize the distribution ( $\chi^2 = 10.4$ , 4 df,  $p = 0.03$ ). In Figure 3D, the plot of MN on age was given. The slight increase observed was not significant. However, an age effect could reasonably be suspected because the slope was higher than that in Porto Tolle and the age was significantly higher ( $t = 2.08$ ,  $p = 0.039$ ) as well. In Figure 4A, we report the

distribution of mean SCE/chromosome regarding 27 subjects after *in vitro* lymphocyte treatment with DEB. The distribution deviated from normality ( $\chi^2 = 3.62$ , 1 df,  $p = 0.05$ ) for the presence of at least two individuals who showed high SCE inducibility. In Figure 4B, the possible correlation between spontaneous and induced SCE was illustrated. Although a positive trend was visible, no statistical significance has been reached so far because of the limited number of tested subjects and the presence of three subjects with low spontaneous levels and high inducibility. Our data generally agree with those of Wiencke et al. (3), although the proportion of high responsive subjects was lower. The limited number of subjects analyzed so far do not allow adequate comparison.

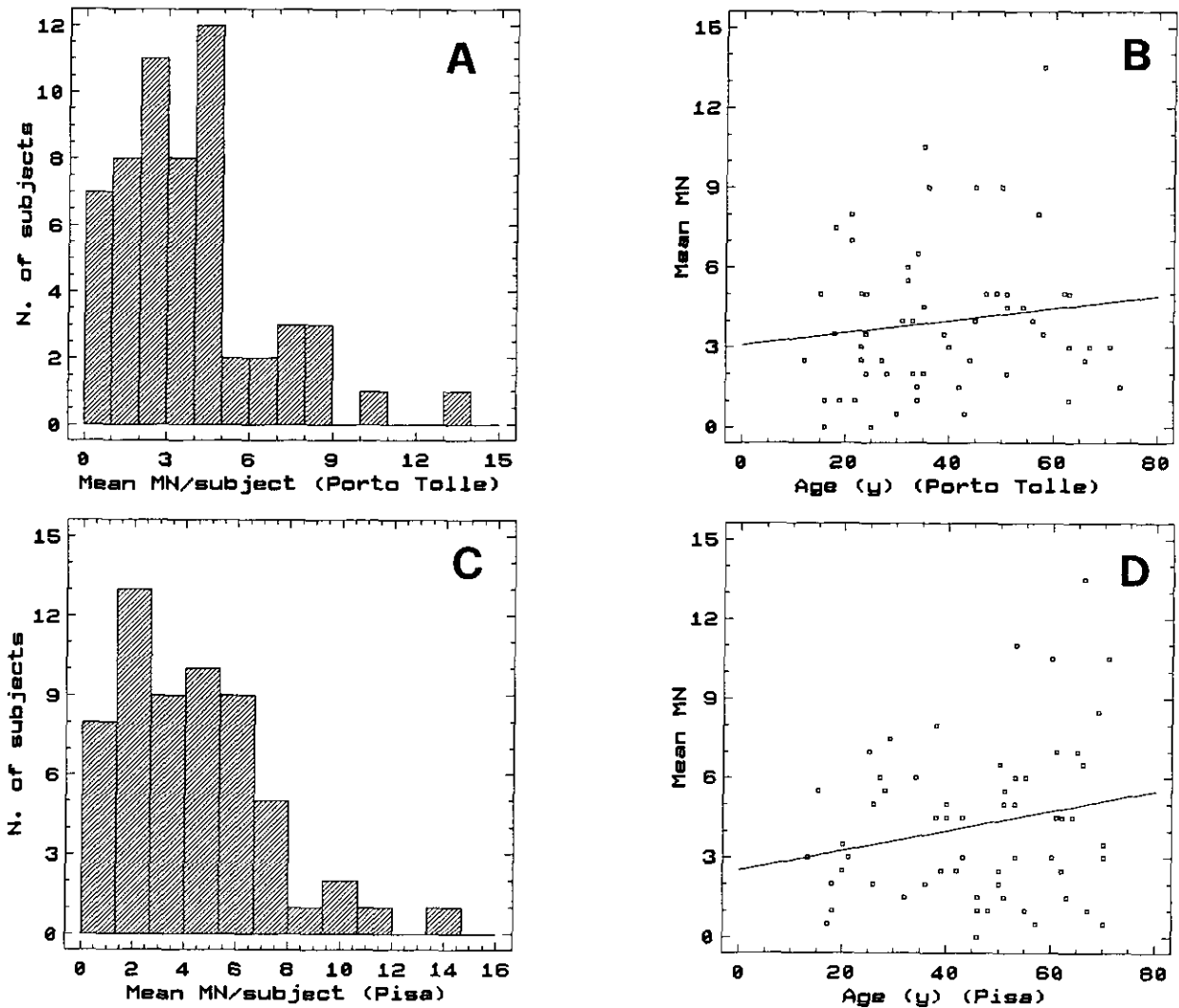


FIGURE 3. Distribution of individuals according to mean micronuclei (MN) in the Porto Tolle sample (A) and in the Pisa sample (C). Plot of mean MN on age: Porto Tolle sample (B) and Pisa sample (D).

## Discussion

From the preliminary evaluation of the data obtained in the first part of the project, several interesting conclusions can be made. The standardized protocol seems to assure a good homogeneity in methods and comparability of results throughout the study. This will allow several correlation studies between the cytogenetic data and all the other variables considered in the project. The differences found between the two populations and among their subsets are suggestive of possible interesting findings. The Porto Tolle sample, in spite of the lower average age, showed a significantly higher SCE frequency than the Pisa sample. Sex and smoking habit, known to influence SCE (7-9), were equally represented in both samples; therefore we suggest that other environmental factors be involved.

In Porto Tolle sample, the effect of smoking was much more evident in females than in males. The amount and

types of cigarettes were similar in both the subsets; therefore, other factors, such as diet and air pollution levels, will be included in further correlation analysis.

As seasonal variations in SCE and CA frequencies have been recently demonstrated by Anderson et al. (10), we considered this variable as well. In Figure 5A and B, we report the SCE frequency distribution observed in the two populations according to the sampling months. There was a statistically significant increase of SCE from January to April in the Porto Tolle sample ( $t = 5.7$ ,  $p < 0.0001$ ), whereas in Pisa from June to September no variation was observed ( $t = 0.27$ ,  $p = 0.78$ ). We thought it was too early to exhaustively study this phenomena, and further analyses in following years have been requested. The DEB test showed that there was a wide SCE response variability, indicating the presence of individuals with different sensitivity to the mutagen as previously reported by other investigators (3). Because data were collected on a house-

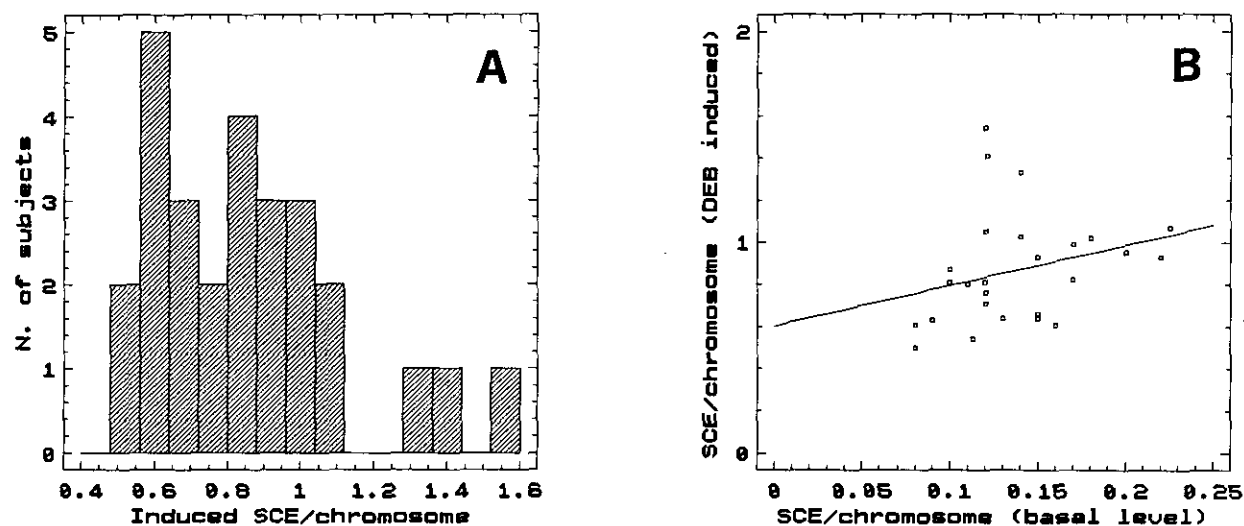


FIGURE 4. *In vitro* test with diepoxybutane (DEB). Distribution of 27 individuals from Pisa population regarding sister chromatid exchanges (SCE)/chromosome (A). Correlation between spontaneous and DEB induced SCE/chromosome in the same subset (B).

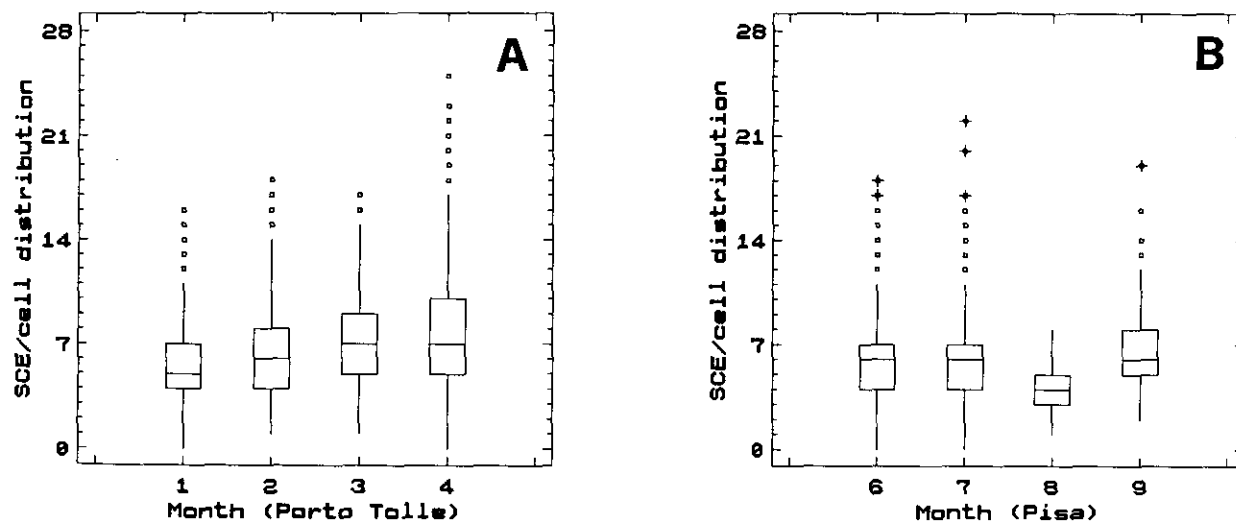


FIGURE 5. Multiple box and whisker plot of sister chromatid exchanges/cell distribution according to sampling period (by month) in the two populations: (A) Porto Tolle and (B) Pisa. Points between 1.5 and 3.0 interquartile ranges from the first or third quartile appear as small open boxes, whereas points beyond 3.0 interquartile ranges appear as a cross.

hold basis, it would be possible to verify whether genetic makeup was involved in such different sensitivities in addition to unknown environmental factors.

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